

Isolation of alpaca anti-idiotypic heavy chain single domain antibody for the aflatoxin immunoassay

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Materials. All reagents were of analytical grade unless otherwise specified. Anti-aflatoxin monoclonal antibody (MAb) 1C11 was produced in our laboratory¹. Aflatoxin B₁, B₂, G₁, G₂, M₁ standard, bovine serum albumin (BSA), polyethylene glycol 8000 (PEG 8000), Tween 20, Freund's incomplete adjuvant, and 3, 3', 5, 5'-tetramethylbenzidine (TMB) were obtained from Sigma (St. Louis, MO, USA). E. coli ER2738 competent cells from the ER2736 line of E. coli were purchased from Lucigen Corp. (Middleton, WI, USA), Top 10F' competent cells and SYPRO Ruby, and Superscript III First-Strand Synthesis system were purchased from Life Technologies (Grand Island, NY). Mouse anti-M13 monoclonal antibody conjugated to horseradish peroxidase (HRP) was purchased from GE Healthcare (Piscataway, NJ, USA). Helper phage M13KO7 and SfiI were obtained from New England Biolabs (Ipswich, MA, USA). QIAprep Spin MiniPrep Kit, QIAquick Gel Extraction Kit and QIAquick PCR Purification Kit were all from Qiagen. B-PER protein extraction reagent, HisPur Ni-NTA Resin, BCA protein assay kit and Nunc MaxiSorp flat-bottom 96 well plates were purchased from Thermo Fisher Scientific Inc. (Rockford, IL, USA). LeukoLOCK total RNA isolation system was obtained from Applied Biosystems (Foster City, CA). pComb3X phagemid vector was a generous gift from Dr. Carlos F. Barbas (The Scripps Research Institute, La Jolla, CA).

Immunization. A 4-year old neutered male alpaca was immunized subcutaneously with 200 µg of anti-aflatoxin MAb 1C11 mixed with Freund's incomplete adjuvant. Three additional injections were given at 2-week intervals. After the last booster, 2×10 mL blood was used for mRNA extraction.

Construction of phage-displayed VHH library. The total RNA was extracted from alpaca blood with the LeukoLOCK total RNA isolation system following the manufacturer's protocols. cDNA was generated using Superscript III First-Strand Synthesis system. DNA fragments

encoding the VHH variable domains were amplified by PCR using a previously published procedure.² PCR products were purified with QIAquick Gel Extraction Kit and digested with SfiI restriction enzyme. Amplified fragments were pooled and ligated into the pComb3X phagemid vector at 1:3 molar ratio. Recombinant plasmids were introduced into competent cells of strain ER2738 of *E. coli* cells by 10 electroporation. After amplification of the cells and the addition of helper phage, the phage-displayed VHH library was harvested as described previously.² Immediately following the transformation and addition of SOC medium, a small aliquot of the electroporated cells was serially diluted and pooled on LB agar plates to determine the library size. The resulting library had an estimated size of 1.12×10^9 independent clones.

Panning and selecting of anti-idiotypic VHH by phage ELISA. Three wells of a microtiter ELISA plate were coated with purified MAb 1C11 (10 µg/mL) in 100 µL of phosphate-buffered saline (PBS) at 4 °C overnight. Nonspecific binding was blocked by incubation with 300 µL PBS containing 3% BSA for 1 h at 37 °C. To prevent selection of BSA-binding phages, 3 wells of a 96-well plate coated with 100 µL 3% BSA in PBS were used for phage pre-absorption. For the panning-elution procedure, the antibody library (1.8×10^{12} pfu/mL) diluted with PBS containing 1% BSA was first added to the pre-absorption wells and incubated at 37 °C for 1 h. Then the supernatant was transferred to the antibody-coated wells and incubated with shaking at room temperature for 2 h. The wells were washed 10 times by PBS containing 0.1% (v/v) Tween 20 (PBST). The bound phages were eluted by using 100 µL of 100, 10, 1 and 0.1 ng/mL AFB₁ in 10% methanol/PBS in the four rounds of panning, respectively, with shaking for 30 min at room temperature. The elution solution was then collected and used to infect *E. coli* ER2738 for amplification and titration. The amplified phage was used for each subsequent round of panning.

After four rounds of panning-elution selection, individual clones were selected from LB-carbenicillin plates and cultured overnight. The isopropyl β -D-1-thiogalactopyranoside (IPTG)-induced supernatants were mixed equally with 50 μ L 10% methanol/PBS with 100 ng/mL AFB₁ or pure dilution buffer and added to a plate containing monoclonal antibody (MAb) 1C11 at 1 μ g/well. A nonspecific binding detection was performed by adding the mixture to wells containing only BSA which was used as a blocking buffer. After 1 h of incubation and 10 times washing with 0.05% PBST, the binding phage were detected using an anti-M13 antibody-HRP. Plasmid DNAs from the positive clones were extracted and sequenced using the primer gback (GCCCCCTTATTAGCGTTTGCCATC) (Division of Biological Sciences, Automated DNA Sequencing Facility, University of California, Davis).

Expression and purification of VHHs. Phagemids from unique sequences of the positive clones were transformed into and expressed in non-suppressor E. coli strain TOP10F' cells. For expression, 100 mL SB medium was incubated with an overnight culture of TOP10F' cell carrying VHH expression plasmid and incubated at 37 °C with shaking at 250 rpm. When the culture reached an OD₆₀₀ value of 0.6-0.8, 1 mM of IPTG was added, followed by continuous shaking overnight.

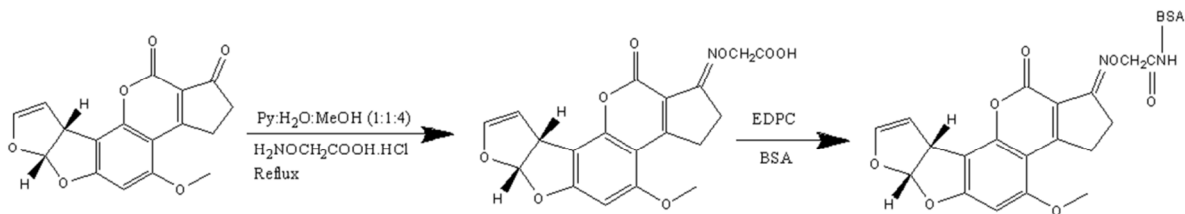
Proteins were isolated using B-PER with protease inhibitors according to manufacturer's instructions. VHH antibodies containing 6xHis tag were purified with Ni-NTA metal affinity chromatography following manufacturer's instruction. The purity of VHHs was assessed using 15% reducing SDS-PAGE according to a standard protocol, followed by staining with SYPRO Ruby. The concentration of VHHs was determined by BCA protein assay kit.

VHH ELISA. An ELISA plate was coated with VHH at 0.45 μ g/well overnight at 4 °C. After blocking with 3% skimmed milk in PBS, 50 μ L of each serial concentration of AFB₁ standard in

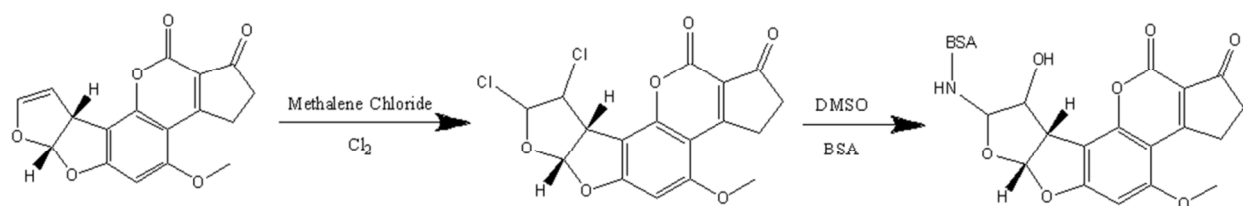
20% methanol/PBS or sample extract was mixed equally with MAb 1C11 in PBS and added to the wells. After 1 h incubation at 37 °C and 3 washing cycles, 100 µL of goat anti-mouse antibody conjugated with HRP was added to each well following by 1 h incubation at 37 °C. The color was developed by adding 100 µL peroxidase substrate (25 mL of 0.1 M citrate acetate buffer [pH 5.5], 0.4 mL of 6 mg/mL TMB in dimethyl sulfoxide [DMSO], and 0.1 mL of 1% H₂O₂) and incubated for 15 min at 37°C. Enzyme reactivity was stopped by adding 50 µL of 2 M H₂SO₄ and the absorbance was detected at 450 nm by a microplate reader.

Sample preparation. Samples of rice, corn, and peanuts obtained from the local grocery store were finely ground using a grinder and stored in the freezer at -20 °C before use. Five grams of each sample was weighed and extracted by 80% methanol in water and incubated at room temperature with shaking at a speed of 250 rpm for 20 min. Then the mixture was centrifuged at 900 g for 30 min and the supernatant was used for sample analysis after dilution.

Statistical analysis. Data were plotted using SigmaPlot version 11.0, from Systat Software, Inc., San Jose California USA, www.sigmaplot.com. Fifty percent inhibitory concentrations of aflatoxin B₁ (IC₅₀) were derived from four parameter logistic regression.



Scheme S-1. Synthesis of AFB₁ oxime-BSA conjugate



Scheme S-2. Synthesis of AFB₁Cl₂-BSA conjugate

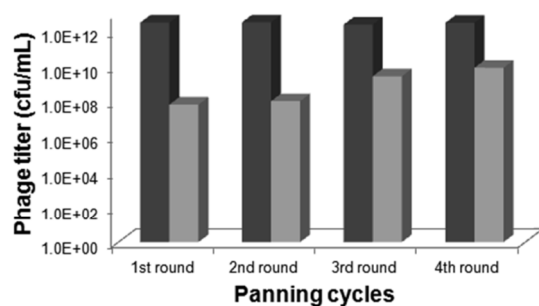


Figure S-1. Number of phage input (dark bars) and output (light bars) of each panning cycle.

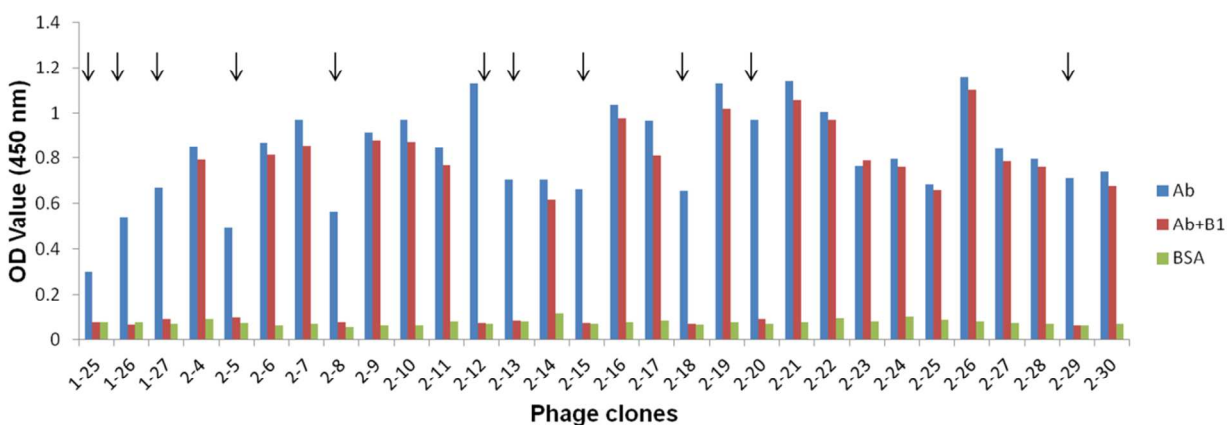


Figure S-2. Phage ELISA results with 30 single clones towards MAb 1C11 with aflatoxin B₁ (red column) or without aflatoxin B₁ (blue column). The binding activity with BSA (green column) was tested to determine the non-specific binding. The arrows indicate positive VHH clones that interfere with aflatoxin binding to the antigen binding site of the MAb 1C11.

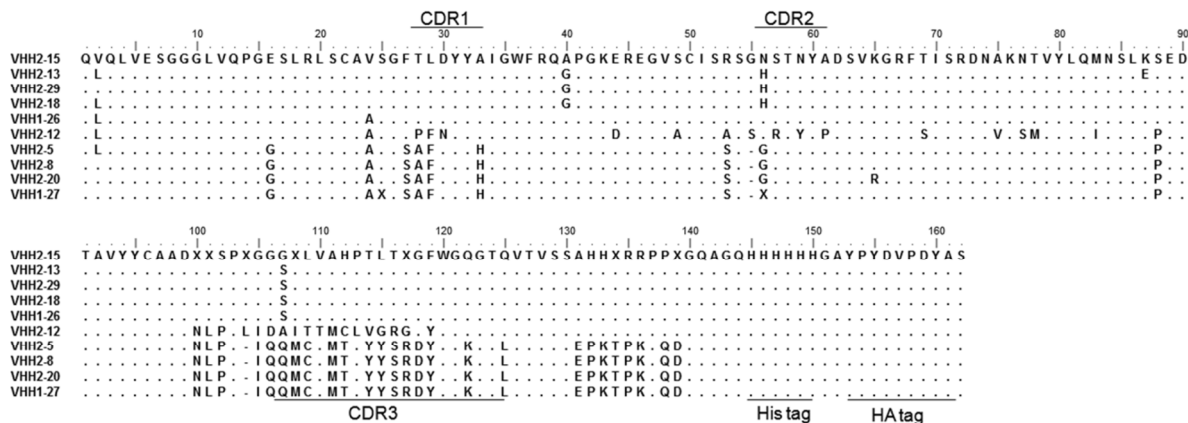


Figure S-3. Alignment of amino acid sequences of selected VHs. Only amino acid residues different from the top one (VHH 2-15) at the same position have been indicated. Dots indicate identity to the top sequence and dashes indicate absence of amino acid residues compared to the longest sequence.

Table S-1 Matrix effect measured as % color reduction and IC₅₀ values^a

| Sample | 4 × dilution | | 8 × dilution | | 20 × dilution | |
|--------|--------------|------------------|--------------|------------------|---------------|------------------|
| | % color | IC ₅₀ | % color | IC ₅₀ | % color | IC ₅₀ |
| | reduction | | reduction | | reduction | |
| Peanut | 18.8% | 0.414 | 13.8% | 0.241 | 10.21% | 0.327 |
| Corn | 16.2% | 0.336 | 17.4% | 0.300 | 14.1% | 0.220 |
| Rice | 14.4% | 0.481 | 14.4% | 0.515 | 12.9% | 0.515 |

^a 20% methanol-PBS was used for the standard assay. The sample extract was diluted 1 to 4 with PBS (the final methanol concentration is 20% in the extract), 1 to 8 with 10% methanol-PBS (the final concentration of methanol in the sample is 18.75%), 1 to 20 with 20% PBS-methanol (final concentration of methanol is 24%). The % color reduction was calculated as $(1 - A_{\text{sample}}/A_{\text{methanol}}) \times 100$, where the A_{sample} is absorbance (450nm) of test sample without aflatoxin and A_{methanol} is absorbance value of 20% methanol-PBS without aflatoxin.

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